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Evaluation of gene expression signatures predictive of cytogenetic and molecular subtypes of pediatric acute myeloid leukemia

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Pediatric acute myeloid leukemia is a heterogeneous disease characterized by non-random genetic aberrations related to outcome. The genetic subtype is currently detected by different diagnostic procedures which differ in success rate and/or specificity.

Design and Methods

We examined the potential of gene expression profiles to classify pediatric acute myeloid leukemia. Gene expression microarray data of 237 children with acute myeloid leukemia were collected and a double-loop cross validation approach was used to generate a subtype-predictive gene expression profile in the discovery cohort (n=157) which was then tested for its true predictive value in the independent validation cohort (n=80). The classifier consisted of 75 probe sets, representing the top 15 discriminating probe sets for *MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)-positive acute myeloid leukemia.

Results

These cytogenetic subtypes represent approximately 40% of cases of pediatric acute myeloid leukemia and were predicted with 92% and 99% accuracy in the discovery and independent validation cohort, respectively. However, for *NPM1*, *CEBPA*, *MLL*(-PTD), *FLT3*(-ITD), *KIT*, *PTPN11* and *N/K-RAS* gene expression signatures had limited predictive value. This may be caused by a limited frequency of these mutations and by underlying cytogenetics. This latter is exemplified by the fact that different gene expression signatures were discovered for *FLT3*-ITD in patients with normal cytogenetics and in those with t(15;17)(q21;q22)-positive acute myeloid leukemia, which pointed to *HOXB*-upregulation being specific for *FLT3*-ITD$^+$ cytogenetically normal acute myeloid leukemia.

Conclusions

In conclusion, gene expression profiling correctly predicted the most prevalent cytogenetic subtypes of pediatric acute myeloid leukemia with high accuracy. In clinical practice, this gene expression signature may replace multiple diagnostic tests for approximately 40% of pediatric acute myeloid leukemia cases whereas only for the remaining cases (predicted as 'acute myeloid leukemia-other') are additional tests indicated. Moreover, the discriminative genes reveal new insights into the biology of acute myeloid leukemia subtypes that warrants follow-up as potential targets for new therapies.

Key words: pediatric AML, gene expression profile, cytogenetic subtype, classification, microarray

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Introduction

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease that accounts for 15-20% of the acute leukemias in children[1] and is classified according to the WHO classification, which is based on non-random genetic aberrations.[2] Over the decades the outcome of pediatric AML has improved and current overall survival rates range from 50% to 70%.[3] The most important prognostic factors in pediatric AML are response to induction therapy and the cytogenetic and molecular subtype of the disease.[4,5]

Gilliland *et al.* postulated that the pathogenesis of AML requires both type I and type II mutations.[6] Type II mutations are often chromosomal rearrangements of transcription factors leading to impaired differentiation of the hematopoietic cell, such as 11q23/*MLL*-rearranged, t(8;21)(q22;q22)[*RUNX1-RUNXT1*], inv(16)(p13q22)[*CBFB-MYH11*], or t(15;17)(q21;q22) [*PML-RARA*]. Patients with t(8;21)(q22;q22), inv(16)(p13q22) and t(15;17)(q21;q22)-positive AML have a favorable prognosis in contrast to *MLL*-rearranged cases. Type I mutations often reflect molecular mutation hotspots in specific genes (*FLT3*, *KIT* and *NRAS*, *KRAS*, *PTPN11* and *NF1*) involved in the proliferation of hematopoietic cells.[7,8] In adult and pediatric AML *FLT3*-internal tandem duplications (*FLT3*-ITD) and *KIT* mutations have been correlated with an inferior outcome.[9-11]

In approximately 20% of pediatric AML cases no chromosomal aberrations have yet been discovered. These patients with apparent cytogenetically normal (CN) AML are currently treated as a homogeneous group with an intermediate risk factor. However, point mutations and small deletions in *CEBPA* and *NPM1* as well as partial tandem duplications in *MLL* (*MLL*-PTD) are found in both pediatric and adult CN-AML. The frequency of these mutations is lower in children than in adults. Moreover, the prognostic impact differs between children and adults.[10,12-15] These observations highlight the genetic heterogeneity within AML as well as between adults and children with AML and the need for separate studies in pediatric AML to demonstrate the value of mutations for stratification in contemporary pediatric AML treatment protocols.

A new case of AML is currently primarily identified by cytomorphology and immunophenotyping. Further characterization needed for risk-stratification includes the detection of chromosomal aberrations by conventional karyotyping and molecular cytogenetics of specific genetic lesions, for instance by fluorescence *in situ* hybridization (FISH) and/or reverse transcriptase (RT) polymerase chain reaction (PCR). However, it can be difficult to obtain a karyogram since this requires successful induction of *in vitro* cellular proliferation to obtain metaphases for analysis of chromosomal changes. In addition, FISH and RT-PCR procedures may also yield inconclusive results, for example due to poor interphase preparations (FISH), limitations in signal detection (FISH), sub-clonality (FISH and RT-PCR) and sequence variations in probe/primer-hybridizing regions (FISH and RT-PCR).

Microarray-based gene expression profiling studies showed that pediatric and adult AML can be accurately classified into cytogenetically distinct subtypes.[16-20] In the Microarray Innovations-in-LEukemia (MILE) study, gene expression profiles accurately classified over 3000 cases with acute and chronic leukemia.[21]

We recently showed that a double-loop cross-validation classification approach yielded a highly stable and accurate classifier with high predictive value for subtypes of pediatric acute lymphoblastic leukemia in both the cross-validation cohort as well as in a totally independent cohort of pediatric ALL.[22] In the current study we used this double-loop cross-validation method to determine whether gene expression signatures can predict prognostically relevant specific cytogenetic subtypes (11q23/*MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22), t(7;12)(q36;p13) and CN-AML) as well as cases with molecular aberrations in *NPM1*, *CEBPA*, *FLT3-ITD*, *N/K-RAS*, *KIT* and *PTPN11* in pediatric *AML*.

Design and Methods

Patients

Viable frozen bone marrow or peripheral blood samples from 237 children with *de novo* AML, 33 with relapsed and 8 with secondary AML were provided by the Dutch Childhood Oncology Group, 'Berlin-Frankfurt-Münster' AML Study Group, Czech Pediatric Hematology and St. Louis Hospital in Paris, France. Informed consent was obtained from patients, after Institutional Review Board approval according to national law and regulations. Leukemic cells were isolated by sucrose density centrifugation and non-leukemic cells were eliminated as previously described.[23] All processed samples contained more than 80% leukemic cells, as determined morphologically using cytospins stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Subsequently, at least 5×10^6 leukemic cells were lysed in Trizol reagent (Gibco BRL, Life Technologies, Breda, the Netherlands). Genomic DNA and total RNA were isolated according to the manufacturer's protocol, with minor modifications.[24]

Cytogenetics

Leukemic samples were routinely investigated for cytogenetic aberrations by standard chromosome-banding analysis, and screened, by the above mentioned study groups, for recurrent non-random genetic aberrations characteristic of AML as described by the WHO 2008 classification of myeloid neoplasms and acute leukemia,[2] including *MLL*-rearrangements, inv(16)(p13q22), t(8;21)(q22;q22) and t(15;17)(q21;q22), using RT-PCR and/or FISH. In the case of incomplete data, the Erasmus MC group performed RT-PCR to detect inv(16)(p13q22), t(8;21)(q22;q22) and t(15;17)(q21;q22) and split-signal FISH to detect rearrangements of the *MLL*-gene using standardized primers and probe combinations, as previously described.[25,26] In three cases, predicted as *MLL*-rearranged AML, screening for an *MLL*-rearrangement was performed with long-distance inverse (LDI) PCR as previously described.[27] In addition, all patients under the age of 18 months were screened for t(7;12)(q36;p13) by FISH. The probes used were five cosmid clones covering the breakpoints in the *ETV6* gene and a PAC clone (RP5-1121A15) containing the *HLXB9* gene, as previously described.[28]

Mutation analysis

Samples were screened for hotspot mutations in *NPM1*, *CEPBA*, *FLT3*-ITD, *NRAS*, *KRAS*, *PTPN11*, *KIT* and *MLL*-PTD, as previously described.[7,29-32] If positive for *MLL*-PTD, this was confirmed by a multiplex ligation-dependent probe amplification (MLPA) analysis (MRC Holland, Amsterdam, the Netherlands). The reaction mix for MPLA-analysis contained probes for exon 2 to 13 of *MLL* for *MLL*-PTD detection and exon 17 of *MLL* as an internal control. A probe in the *serpinB2* gene was used as an external control.[33] Data were analyzed using GeneMarker v1.5 (Softgenetics, State College, USA).

Microarray

The integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, USA). cDNA and biotinylated cRNA were synthesized and hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, USA) according to the manufacturer's guidelines. Data were acquired using 'expresso' (Bioconductor package 'Affy'), and probe-set intensities were normalized using the variance stabilization normalization procedure (Bioconductor package 'VSN') in the statistical data analysis environment R, version 2.2.0. The original data files have been submitted to the GEO database (GSE17855).

Statistics

To find signatures for the different cytogenetic and molecular subtypes an empirical Bayes linear regression model was used to compare samples from each group to all other samples.[34] This model takes advantage of the large number of probe sets to yield better estimates for the gene-specific standard error, producing more powerful tests for differential expression even if small sample sizes are involved. Moderated t-statistics *P* values were corrected for multiple testing using the false discovery rate (FDR) method defined by Benjamini and Hochberg.[35] The top 50 most significant probe sets for each subtype were used as a starting point to construct the classifier.

The construction of the classifier

Samples were divided at random into a discovery cohort of 157 cases that were used for the double-loop cross validation approach and an independent cohort of 80 cases, which was only tested once and served as a true independent validation cohort. In all following 100 cycles of the double-loop cross-validation approach, the sample distribution of the discovery cohort reflected the distribution of cytogenetic subtypes as seen in the total cohort of 237 cases. The double-loop cross-validation method was used to build a support-vector machine-based classifier predictive for the known cytogenetic subtypes of pediatric AML. This approach avoids over-fitting of gene expression profiling data[36] and has proven to yield a stable classifier with high accuracy to predict subtypes of pediatric acute lymphoblastic leukemia, as we previously showed.[22] The double-loop cross-validation method was only applied to the discovery cohort of 157 cases. This consists of an inner loop containing two-thirds of cases in which the minimal number of probe sets yielding the highest prediction sensitivity is being determined (100 iterations for each selected number of probe sets) and an outer loop containing the remaining one-third of cases serving to validate the obtained results from the inner loop (also 100 iterations per list of probe sets) (*Online Supplementary Figure S1*).

In each of 100 runs of the inner loop, patients were randomly assigned to the inner-training (9/10) and inner-test (1/10) group (10-fold cross-validation). To start, the top 50 probe sets most discriminative for each subtype were selected by rank of *P* values obtained by applying an empirical Bayes linear regression model (LIMMA) to the inner-training group. These probe sets were used to construct a support vector machine-based classifier which was then tested for predictive sensitivity on the inner-test group of the remaining 1/10 of cases (100 iterations). Next, the minimum number of probe sets that optimally classified the patients in the inner loop was obtained by backwards selection starting with 250 probe sets (50 probe sets x 5 subtypes) using a global test for ranking the significance of probe sets in each iteration in order to reduce multiple testing errors, as previously described.[22] The optimal number of probe sets determined in the inner loop was used to construct a classifier for which the median sensitivity was estimated via 3-fold cross-validation by applying the trained classifier to the remaining one-third of the cases of the outer loop (100 iterations; *Online Supplementary Figure S1*). The final gene expression classifier, trained on all 157 cases in the discovery cohort, was used to determine the prediction accuracy in the independent group of 80 cases (*Online Supplementary Figure S1*).

The same approach was used to select probe sets predictive for the most frequent molecular aberrations, i.e. *NPM1*, *CEBPA*, *MLL*-PTD, *FLT3*-ITD, *KIT*, and combined mutations in the *RAS*-pathway (*NRAS*, *KRAS* and *PTPN11*). Since *NPM1*, *CEBPA* and *MLL*-PTD were mutually exclusive from the other cytogenetic subgroups, these abnormalities could be simultaneously included in one model together with the known cytogenetic subgroups, for which the prediction accuracy was estimated as described above. In addition, we performed an analysis in which the most discriminative probe sets for type I mutations in *FLT3*-ITD, *KIT* and *RAS*-pathway were identified, after adjusting for the underlying cytogenetic aberrations.

Software

R (version 2.2.0 and version 2.5.0) and the R packages affy, vsn, e1071, globaltest, limma, multtest and marray were used to run the above-mentioned analyses.[34,37-41] Hierarchical clustering analysis was performed in Genemaths XT (Applied Maths, Austin, USA).

Results

Patients' characteristics

Gene expression profiles were generated from 237 newly diagnosed pediatric AML cases. Non-random cytogenetic subgroups of pediatric AML with a sufficient number of cases were included, i.e. *MLL*-rearranged AML, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and CN-AML. In addition, seven t(7;12)(q36;p13)-positive infant AML cases were included (Table 1). For the other cytogenetic groups, e.g. t(6;9)(p23;q34) (n=7), monosomy 7 (n=4), trisomy 8 (n=1) and complex karyotype (n=11), no significant discriminative genes were found and these cases were, therefore, combined into a single group annotated with 'remaining cytogenetics'. No karyotype was available for 25 cases but since these cases were negative for *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13), these cases were included in the 'unknown other cytogenetics' category.

Definition of subgroups

Using an empirical Bayes linear regression model many discriminative probe sets, with high statistical significance ($P<1.0^{-08}$), were found for *MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)-positive AML (*Online Supplementary Table S2*). In contrast, only a limited number of discriminative probe sets were found to be significant at lower *P* value ($P<1.0^{-04}$) for CN-AML, cases with remaining genetic aberrations or unknown other cytogenetics (*Online Supplementary Table S2*). Hereafter, this mixed group is referred to as the 'AML-other' group. Based on an equal distribution of these groups, the overall cohort was divided into a discovery cohort (n=157) to construct the classifier and an independent validation cohort (n=80).

Probe set selection for classifier estimated with the discovery cohort

The classifier was constructed by selecting the most statistically significantly discriminative probe sets for each of the five cytogenetic subtypes: *MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)-positive AML. A double-loop cross validation approach was used that also included a backward selection procedure to keep the number of probe sets needed for most accurate classification to a minimum in order to avoid over-fitting of data, as previously described.[22] In the inner-loop the minimum number needed for the highest predictive sensitivity of 100% was determined to be 75 probe sets (Table 2, *Online Supplementary Table S3*), i.e. 15 probe sets per cytogenetic subtype, whereas randomly selected probe sets only yielded a median sensitivity of 60% (*Online Supplementary Figure S2*). Some of these probe sets represented the same gene, e.g. four probe sets presented *RUNX1T1* for the t(8;21)(q22;q22) subtype (Table 2). The constructed classifier took into account the expression levels of all 75 probe sets, including the 60 probe sets that were not selected for a particular subtype. The classifier built with these 75 probe sets yielded a median accuracy of 92% in the outer loop. Notably, all inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13)-positive cases were correctly predicted in each of the 100 iterations (100% sensitivity, specificity, positive predictive value and negative predictive value) (Table 3A).

Hierarchical clustering showed that the cytogenetic subtypes formed distinct clusters according to the gene expression signature using these 75 probe sets (Figure 1A, *Online Supplementary Table S3*). Only three AML-other patients were misclassified as having *MLL*-rearranged AML. In these cases, *MLL* involvement could not be confirmed with FISH, but LDI-PCR revealed that all three samples did indeed harbor an *MLL*-rearrangement (*Online Supplementary Table S4*). These three samples were, therefore, included as true positive *MLL*-rearranged cases in the construction of the final classifier in the discovery cohort. As expected, including these cases as *MLL*-rearranged AML improved the diagnostic values of the 3-fold cross validation in the outer loop (Table 3B).

Independent validation of the classifier

The true accuracy of the classifier was tested in the independent validation cohort of 80 patients. The true sensitivity, specificity, positive predictive value, negative predictive value and accuracy in this validation cohort was 98%,

Table 1. Cytogenetic and molecular characteristics of the pediatric acute myeloid leukemia patients in this study.

	Discovery cohort (n=157)		Validation cohort (n=80)		Total (n=237)	
	N	%	N	%	N	%
Cytogenetic subtypes						
MLL-rearrangements	31	20	16	18	47	20
t(8;21)(q22;q22)	18	11	10	11	28	12
inv(16)(p13q22)	17	11	10	11	27	11
t(15;17)(q21;q22)	14	9	5	6	19	8
t(7;12)(q36;p13)	5	3	2	2	7	3
AML-other[#]						
CN-AML[a]	24	15	15	17	39	16
Remaining cytogenetics[a]	33	21	12	14	45	19
Unknown other cytogenetics[a]	15	10	10	11	25	11
Molecular subtypes						
NPM1[b]	9	5	8	9	18	7
MLL-PTD[b]	3	2	3	3	6	2
CEBPA[b]	10	6	6	7	16	6
FLT3-ITD	30	18	18	21	48	19
KIT	12	7	6	7	18	7
N/KRAS	23	14	18	21	41	16
PTPN11	4	2	1	1	5	2

[a]Including three cases predicted as MLL-rearranged AML and confirmed by LDI-PCR; [#]Together forming the AML-other subtype used throughout this classification study. All samples were negative for MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13). [b]These subtypes were only observed in samples in the AML-other subtype.*

Table 2. Overview of discriminative genes used in the classification of pediatric acute myeloid leukemia.

MLL-rearranged AML	t(8;21)(q22;q22)	inv(16)(p13q22)	t(15;17)(q21;q22)	t(7;12)(q36;p13)
WHAMML1 (2)	*RUNX1T1* (4)	*MYH11* (3)	*HGF* (2)	*TP53BP2*
ITM2A (2)	*IL5RA* (2)	*LPAR1* (2)	*STAB1* (2)	*chr14q23.1*
C10orf140 (2)	*POU4F1* (2)	*NT5E* (2)	*FAM19A5* (2)	*DYX1C1*
C10orf114	*SIPA1L2* (2)	*NRP1*	*ANXA8*	*EDIL3*
CES1	*TRH*	*TM4SF1*	*LGALS12*	*LIN28B*
TBC1D12	*PGAM5*	*LRP4*	*SIX3*	*BAMBI*
PHACTR3	*SIPA1L2*	*CLIP3*	*PGBD5*	*MAF*
LOC84989	*CACNA2D2*	*MN1*	*C2orf82*	*FAM171B*
ZNF91	Unknown (chr8q21.3) (2)	*SPARC*	*FGF13*	*AGR2*
ZNF329		*AK5*	*MST1*	Unknown (chr2q14.3)
Unknown (chr13q22.1)		Unknown (chr17p13.3)	*TNFRSF4*	*CRISP3*
Unknown(chr10p12.31)			*IGDCC4*	*MNX1*
				CTTNBP2
				KRT72
				MMP

In parentheses the number of probe sets representing the same gene. In total 75 probe sets were included in the classifying model, which represents 59 unique genes.

100%, 100%, 97% and 99%, respectively (Table 3C). Only one *MLL*-rearranged AML case was misclassified as AML-other (Table 4). Hierarchical cluster analysis also demonstrated the discriminative value of the selected probe sets in the independent validation cohort (Figure 1B). At this point, only patients at initial diagnosis of AML had been included. Next, we addressed whether the classifier was also suitable for predicting the subtype of 33 relapsed and eight secondary AML cases. All nine *MLL*-rearranged cases (3 secondary and 6 relapsed AML cases), all five t(8;21)(q22;q22) relapsed cases and all 27 other relapsed and secondary AML cases were correctly predicted by our classifier (*Online Supplementary Table S5*).

Table 3. Diagnostic test values for the classification of pediatric acute myeloid leukemia by a gene expression signature consisting of 75 probe sets.

	Discovery cohort[a] 3-fold cross-validation (100 iterations)				
A	% sensitivity	% specificity	% PPV	% NPV	% accuracy
MLL-rearranged	80 (70-90)	95 (95-98)	83 (78-89)	95 (93-97)	93 (92-95)
t(8;21)(q22;q22)	100 (83-100)	100 (100-100)	100 (100-100)	100 (98-100)	100 (98-100)
inv(16)(p13q22)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(15;17)(q21;q22)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(7;12)(q36;p13)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
AML-other	92 (92-96)	92 (88-96)	92 (88-96)	93 (92-96)	92 (90-94)
All groups	92 (88-96)	92 (92-96)	93 (92-96)	92 (88-96)	92 (90-94)

	Discovery cohort[a] 3-fold cross-validation (100 iterations)				
B	% sensitivity	% specificity	% PPV	% NPV	% accuracy
MLL-rearranged#	90 (82-100)	97 (97-100)	92 (89-100)	98 (95-100)	96 (94-98)
t(8;21)(q22;q22)	100 (83-100)	100 (100-100)	100 (100-100)	100 (98-100)	100 (98-100)
inv(16)(p13q22)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(15;17)(q21;q22)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
t(7;12)(q36;p13)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)	100 (100-100)
AML-other	96 (96-100)	94 (89-96)	94 (88-96)	96 (96-100)	96 (92-98)
All groups	94 (89-96)	96 (96-100)	96 (96-100)	94 (88-96)	96 (92-98)

(A) and (B) [a]Values represent the median and 25th-75th percentiles (in parentheses) obtained by 3-fold cross-validation using the discovery cohort of 157 cases (100 iterations). #In contrast to Table 3A, the MLL-*rearranged category in Table 3B now includes the three novel* MLL-*rearranged cases that were predicted by gene expression profiling and confirmed by LDI-PCR.*

	Validation cohort independent validation group, N=80				
C	% sensitivity	% specificity	% PPV	% NPV	% accuracy
MLL-rearranged	94	100	100	98	99
t(8;21)(q22;q22)	100	100	100	100	100
inv(16)(p13q22)	100	100	100	100	100
t(15;17)(q21;q22)	100	100	100	100	100
t(7;12)(q36;p13)	100	100	100	100	100
AML-other	100	98	97	100	99
All groups	98	100	100	97	99

Comparison with other gene expression profiles in pediatric and adult acute myeloid leukemia

Ross *et al.* demonstrated that children with AML could be classified using gene expression profiles generated by Affymetrix U133A microarrays containing 22,283 probe sets.[17] An overall accuracy of 93% was achieved using 150 probe sets to classify *MLL*-rearranged AML, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and acute megakaryoblastic leukemias (M7). In that study, t(7;12)(q36;p13)-positive AML cases were not included. The 150 probe sets of Ross *et al.* were used to construct a classifier in our discovery cohort which was then applied to the independent validation cohort, exactly as done for testing our own 75 probe set-based classifier. Five out of 80 patients were misclassified, yielding an overall predictive accuracy of 94% for Ross' set compared to 99% for the 75 probe sets selected in this study (*Online Supplementary Table S6*). The misclassified cases included two *MLL*-rearranged and two t(7;12)(q36;p13)-positive cases which were assigned to the AML-other category and one AML-other case which was predicted as an MLL-rearranged case. Since only 40 out of our 75 probe sets were present on the U133A microarray used by Ross *et al.*, the reciprocal comparison of our list of selected probe sets on Ross' dataset was not informative.

Valk *et al.* described 16 different subgroups in adult AML using 2,856 probe sets present on Affymetrix U133A microarrays.[18] A classifier built with these 2,856 probe sets resulted in an overall accuracy of 94% when applied to our independent pediatric validation cohort (*Online Supplementary Table S6*). Three *MLL*-rearranged cases and one t(7;12)(q36;p13)-positive case were misclassified as AML-others. One AML-other patient was misclassified as having *MLL*-rearranged AML.

Potential type II molecular aberrations: NPM1, CEBPA and MLL-PTD

Mutations in *NPM1* and *CEBPA* and partial tandem duplications in *MLL* (*MLL*-PTD) might be considered as various type II mutations and were only observed in samples belonging to the AML-other group (*Online Supplementary Table S7*). Moreover, these molecular abnormalities were mutually exclusive reflecting heterogeneity

Table 4. Prediction of the classifier on the independent validation cohort.

	Subtype according to cytogenetic screening					
	MLL	t(8;21)	inv(16)	t(15;17)	t(7;12)	AML Other
SVM predicted subtype						
MLL-rearranged	15	0	0	0	0	0
t(8;21)(q22;q22)	0	10	0	0	0	0
inv(16)(p13q22)	0	0	10	0	0	0
t(15;17)(q21;q22)	0	0	0	5	0	0
t(7;12)(q36;p13)	0	0	0	0	2	0
AML-Other	1	0	0	0	0	37

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy were 98% (51/52), 100% (37/37), 100% (52/52), 97% (37/38) and 99% (79/80), respectively.

among the AML-other cases. For *CEBPA*, 852 probe sets were found to be statistically discriminative between mutated and germ-line cases whereas only 12 probe sets were found to be discriminative for *MLL*-PTD at the same cut-off value of $P<0.05$ (FDR-corrected; *Online Supplementary Table S8*). Three-fold cross validation with the top 15 most discriminative probe sets for mutations in *NPM1*, *CEBPA* and *MLL* (i.e. *MLL*-PTD) revealed a median sensitivity and accuracy in the outer loop of 43% and 92%, respectively, and sensitivity, specificity, positive predictive value, negative predictive value and accuracy of 18%, 98%, 75%, 82% and 81%, respectively, in the independent validation cohort (Table 5A, *Online Supplementary Table S9*). In the independent validation cohort 7/8 cases with an *NPM1* mutation, 4/6 cases with a *CEBPA* mutation and all three *MLL*-PTD cases were misclassified. Moreover, when adding these three molecular subtypes to the previously used five cytogenetic subtypes, the accuracy of 99% based on the five cytogenetic subtypes dropped to 78% in the validation cohort (*Online Supplementary Table S10*). All misclassified cases were assigned to the AML-other category.

Type I mutations: FLT3-*ITD*, KIT, N/K-RAS *and* PTPN11

Internal tandem duplication in *FLT3* (*FLT3*-ITD), mutations in *KIT* and mutations in genes involved in the RAS-pathway [*NRAS* (n=34), *KRAS* (n=7) and *PTPN11* (n=5)] were observed in 44% of all cases. In contrast to *FLT3*-ITD and *KIT* aberrations, no discriminative probe sets were found for *N/K-RAS* and only a limited number for *PTPN11* (*Online Supplementary Table S8*). Combining the aberrations in the RAS-pathway into one group still did not identify discriminative probe sets. We, therefore, only included *FLT3*-ITD and *KIT* into a classification model for the prediction of type I mutations. However, the 30 most discriminative probe sets for these subtypes resulted in a classifier with limited predictive value. The highest predictive values were found for *FLT3*-ITD, with a positive predictive value and negative predictive value of 100% and 93%, respectively (Table 5B, *Online Supplementary Table S11*). Inclusion of the top 15 discriminative probe sets for aberrations in the RAS-pathway (although with $P>0.05$) did not result in prediction of this subtype (*Online Supplementary Table S12*).

Table 5. Diagnostic test values for the prediction of mutations in *NPM1*, *CEBP*· and *MLL*-PTD for the independent validation cohort by the gene expression signature consisting of 45 probe sets (A) and for the prediction of type I molecular subtypes for the independent validation cohort by the gene expression signature consisting of 30 probe sets (B).

	Validation cohort independent validation group, N=80				
A	% sensitivity	% specificity	% PPV	% NPV	% accuracy
NPM1	13	99	50	91	90
MLL-PTD	0	100	ND	96	96
CEBPA	33	100	100	95	95
Remaining cases	98	18	82	75	81
All groups	18	98	75	82	81
	Validation cohort independent validation group, N=80				
B	% sensitivity	% specificity	% PPV	% NPV	% accuracy
FLT3-ITD	72	100	100	93	94
KIT	33	99	66	95	94
Remaining cases	89	63	86	94	88
All groups	63	89	94	86	88

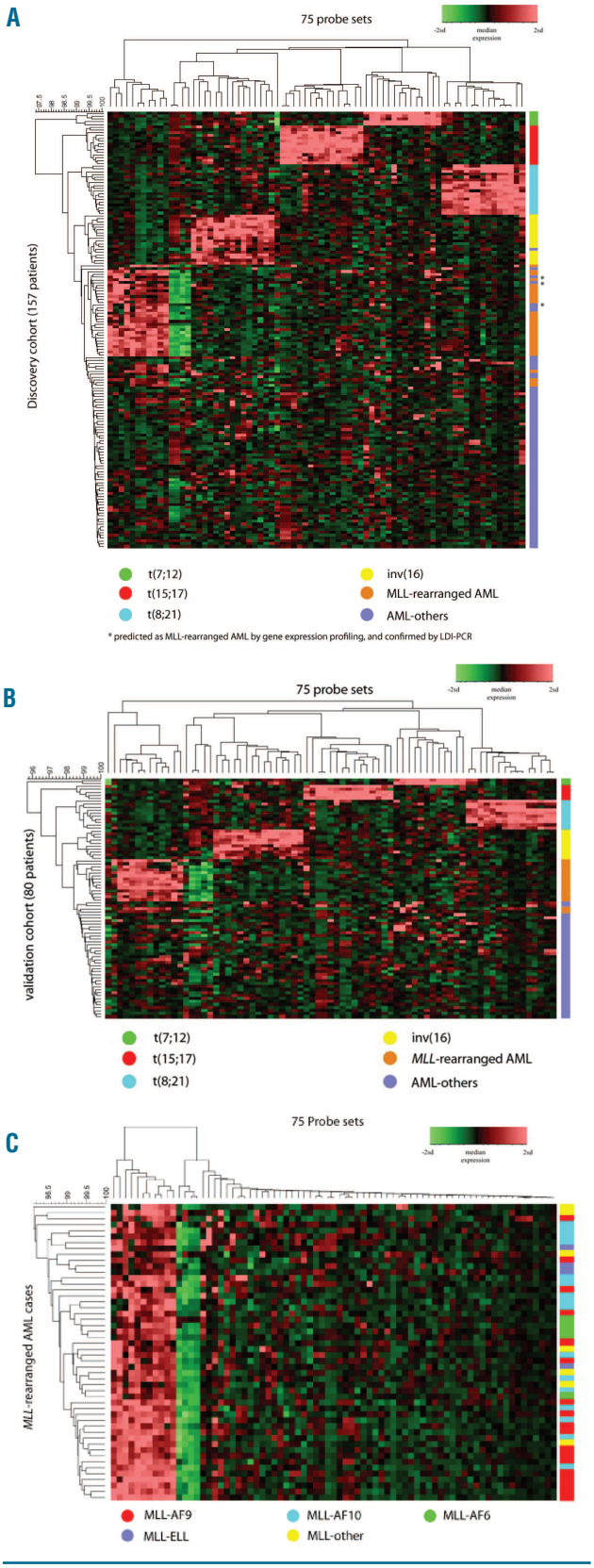

Figure 1. Hierarchical clustering of the cytogenetic subtypes of pediatric AML by gene expression profiling. (A) Hierarchical clustering of 157 patients in the discovery cohort by gene expression signature derived from 75 classifying probe sets (*Online Supplementary Table S2*). (B) Validation of the gene expression pattern in 80 patients of the independent validation cohort (C) *MLL*-rearranged AML cases do not separate into distinct clusters based on similarity in expression pattern related to the translocation partner using the 75 classifying probe sets.

Although a large number of discriminative genes were found for *FLT3*-ITD (*Online Supplementary Table S8*), many probe sets were similar to those found for t(15;17)(q21;q22) instead of being specific for *FLT3*-ITD. This is in line with the fact that *FLT3*-ITD is often found in t(15;17)(q21;q22)-positive cases. To correct for these cytogenetic effects, we applied the Bayes linear regression model while adjusting for cytogenetic subtype. In this multivariate analysis, unique gene expression signatures specific for *FLT3*-ITD-positive cases were found that differ between t(15;17)(q21;q22)-positive and CN-AML cases (Figure 2, *Online Supplementary Table S13*). Specifically, the genes of the *HOXB* cluster were over-expressed in all patients with a *FLT3*-ITD-positive CN-AML and not in *FLT3*-ITD-negative CN-AML or t(15;17)(q21;q22) patients (Figure 2, *Online Supplementary Figure S3*). The same multivariate approach for *KIT* and RAS-pathway mutations did not result in cytogenetic subtype-specific gene expression signatures.

When adding the 15 probe sets discriminative for t(15;17)(q21;q22)/*FLT3*-ITD and the nine most discriminative probe sets [the 6 other probe sets showed overlap with the t(15;17)(q21;q22) subgroup] for CN-AML/*FLT3*-ITD from the multivariate analysis to our classifier, we still could not accurately predict all *FLT3*-ITD cases. Although numbers were small in these subgroups, the accuracy in the independent validation cohort dropped to 86% due to misclassification of cases, especially CN-AML/*FLT3*-ITD cases (*Online Supplementary Table S14*).

Discussion

Cytogenetic aberrations have prognostic value in pediatric AML and, hence, genetic subtypes are used for risk stratification in most current pediatric AML treatment protocols and are part of the current WHO classification of myeloid neoplasms and acute leukemia.[2] In the present study we explored the possibility of microarray-based gene expression profiling to identify the cytogenetic and molecular subtypes in pediatric AML. A gene expression signature of 75 probe sets predicted the most important non-random cytogenetic aberrations in an independent pediatric AML cohort with 99% accuracy and a positive and negative predictive value of 100% and 97%, respectively. In addition, unique gene-expression signatures were found for *FLT3*-ITD AML which differed between cytogenetic subtypes.

Gene expression profiling has been shown to predict the major cytogenetic subgroups in both pediatric and adult AML.[17-19] The sensitivity and specificity of such signatures should be addressed in an independent and representative cohort, since microarray data analysis can easily result in over-interpretation of data.[42] Recently, the MILE study group, using an independent validation cohort of 1,152 cases, robustly showed that gene expression profiles can be used to classify different types of (mainly adult) myelodysplastic syndrome and chronic and acute leukemia cases into known cytogenetic subtypes.[21] This study mainly included adult cases (whose disease may differ in etiology from that of children) and did not address the prediction of molecular abnormalities (e.g. *FLT3*-ITD and *RAS* mutations) by gene expression profiles. In the present study we specifically addressed the value of gene expression profiles for prediction of and classification based on cytogenetic and molecular subtypes of children at initial diagnosis of AML. We previously showed the predictive value of a double-loop cross-validation approach to select classifying probe sets and validated these using an independent validation cohort in pediatric ALL,[22] and therefore used the same unbiased approach in this childhood AML study.

In the present study we identified a gene expression signature of 75 probe sets, representing 15 probe sets for each subgroup, to predict *MLL*-rearranged, t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q21;q22) and t(7;12)(q36;p13) positive AML. When applied to our independent validation cohort the sensitivity, specificity, and negative and positive predictive values of this signature were 98%, 100%, 100% and 97%, respectively. The prediction of *MLL*-rearranged AML, in particular, was better with the newly selected

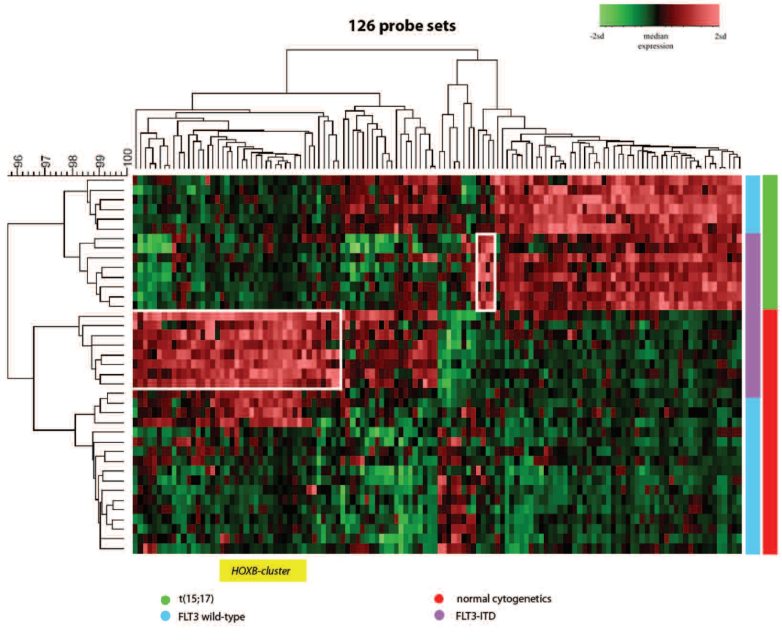

Figure 2. Hierarchical clustering of *FLT3*-ITD positive cases in t(15;17)(q21;q22) and CN-AML. Hierarchical clustering for *FLT3*-ITD in t(15;17)(q21;q22) and CN-AML based on 126 probe sets selected by multivariate analysis including molecular and cytogenetic subtypes (*Online Supplementary Table S12*). Highlighted boxes represent probe sets for *FLT3*-ITD and the specific cytogenetic subtype. The *HOXB* cluster probe sets are represented in yellow.

probe sets than with the previously used probe sets compiled by Ross *et al.*[17] and Valk *et al.*[18] Since 35 probe sets were not present on the Affymetrix U133A microarrays used in both of the former studies, these new probe sets are perhaps decisive for correct prediction of cytogenetic subgroups in pediatric AML.

The 75 probe sets harbored probe sets for genes involved in the specific translocations, e.g. the probe sets for *RUNX1T1/ETO* were highly discriminative for t(8;21)(q22;q22), those for *MYH11* for inv(16)(p13q22) and the probe set for *HLXB9* for t(7;12)(q36;p13). The high expression of these probe sets is probably related to specific hybridization to the fusion transcript, as suggested by Kohlmann *et al.*[43] Remarkably, six out of 15 probe sets discriminative for *MLL*-rearranged AML (Table 2) were located in non-protein coding regions of the genome. Four of these probe sets were located in a relative small (<40 Kb) region on chromosome 10. This is of interest, since nowadays these regions cannot be considered as junk DNA, but might be involved in the regulation of other genes, such as miRNA.[44]

In parallel to the present study we found that expression of one of the discriminative genes for *MLL*-rearranged AML, i.e. brain and reproductive organ-expressed gene (*BRE*), was highly associated with a favorable prognosis in cases with t(9;11)(p22;q23). Functional studies with *BRE* did not reveal that the proliferation, apoptosis or sensitivity towards drugs was altered upon re-expression in AML cells, suggesting that BRE itself has no anti-proliferative function.[45] Besides *BRE*, other new prognostic genes have recently been identified using gene expression profiling. An example is the angiogenic factor *VEGFC*, for which a high level of expression was associated with an unfavorable clinical outcome in both childhood and adult AML.[46] According to gene expression profiling, the *EVI1* gene had no prognostic impact in children with AML, in contrast to the situation in adults, emphasizing the need for separate analysis of pediatric and adult AML.[47] In adult AML gene expression profiling showed that high expression of *ERG* and *MN1* was related to outcome, although their role in malignant transformation remains unknown.[48,49] More recently a unique gene expression signature was identified for the prognostically relevant mutation in *IDH2*, which may help to unravel the role of *IDH2* in the biology of AML.[50] Thus, gene expression profiling identifies new genes linked to subtypes and/or prognosis of AML and may provide important information about the biology of disease when further functional studies have been performed. This knowledge is needed for the rational development and optimization of treatment protocols; moreover, affected genes and pathways may serve as targets for new therapies ('targeted therapy').

Three AML-other cases that were negative by MLL-split signal FISH were initially thought to be misclassified as *MLL*-rearranged AML. However, more detailed analysis of the *MLL*-gene using LDI-PCR confirmed that the *MLL*-gene was indeed rearranged, indicating the higher sensitivity of our gene expression signature than the routine diagnostic FISH procedure in the detection of *MLL*-rearranged cases. Cryptic *MLL*-rearrangements can also be detected using single nucleotide polymorphism (SNP)-array platforms e.g. t(6;11)(q27;q23).[51,52] These finding illustrate the high potential of advanced methods, such as gene expression profiles, LDI-PCR and SNP-arrays, for detecting rearrangements of the *MLL* gene in clinical AML samples.

The cytogenetic subgroups that could be correctly predicted in our study represented approximately 40% of the pediatric AML cases (*Online Supplementary Table S1*). The remaining patients in this study had CN-AML or cytogenetic aberrations other than the five cytogenetic subgroups tested in this study. Recently the subgroup with CN-AML has been further characterized by recurrent molecular aberrations in *NPM1*, *CEBPA* and *MLL* (-PTD). The heterogeneity of pediatric AML is further illustrated by molecular aberrations detected in different cytogenetic subgroups, i.e. *FLT3*-ITD, *KIT* and mutations in the RAS-pathway. Our results show a less accurate prediction of these molecular aberrations by gene expression signatures compared to signatures predictive for cytogenetic subtypes, as was also observed in adult AML.[19,53] Only *FLT3*-ITD could be predicted with a high negative predictive value (93%) and positive predictive value (100%) in the independent validation cohort, but showed lower sensitivity, as described in adult AML cases with *FLT3*-ITD.[54] The low predictive value for the molecular subtypes including mutations in *NPM1*, *MLL*-PTD, *KIT* and *PTPN11* can be explained by the limited number of discriminative probe sets found for each of these aberrations (*Online Supplementary Table S8 versus Online Supplementary Table S2*). This may be because of both limited sample size for each molecular subtype and underlying cytogenetic lesions that have a differential effect on gene expression signatures. A limited sample size itself does not, *per se*, hamper the accuracy of classification if the number of statistically significant probe sets and the fold-change in expression levels of discriminative probe sets is relatively high (exemplified by the high accuracy at predicting t(7;12)(q36;p13)-positive cases despite only five cases being included in the discovery cohort, *Online Supplementary Table S2*). However, in combination with heterogeneity in underlying cytogenetic abnormalities (or other genetic lesions) the number of highly discriminative probe sets becomes limited when sample size is also limited.

Since some of these mutations are not mutually exclusive or are restricted to distinct cytogenetic subtypes, we also selected discriminative probe sets for *FLT3*-ITD using a multivariate approach including cytogenetic subtype. For the other molecular subtypes probe sets could not be identified in a multivariate setting, presumably because of the limited frequency of occurrence of these mutations. Moreover, overlapping signaling pathways between subgroups, no effect of the mutation on transcript level, or different mutations per gene can make it difficult to predict these molecular aberrations correctly by gene expression profiles.[15,55]

Depending on the cytogenetic background, specific genetic aberrations may play different roles in the leukemogenesis of pediatric AML. Interestingly, the genes of the *HOXB* cluster were over-expressed in all patients with a *FLT3*-ITD positive CN-AML, but not in those with a *FLT3*-ITD-positive t(15;17)(q21;q22), which is in concordance with differences in prognostic relevance between these two subgroups. In adult AML, some of these *HOXB* genes were also identified as discriminating genes for patients with a *FLT3*-ITD.[19] In pediatric AML, *HOXB* up-regulation has been correlated with NPM1 mutations in CN-AML.[56] Here we show that *HOXB* over-expression is not restricted to *NPM1*-mutated cases, but is also found in all patients with *FLT3*-ITD-positive CN-AML.

In conclusion, a specific gene expression signature existing of 75 probe sets could accurately identify five cytogenetic subgroups in pediatric AML. Molecular aberrations were hard to predict, which could be due to the low frequency of some of these aberrations and/or gene expression signatures being affected by the underlying cytogenetic abnormality. It remains to be determined whether underlying but yet unknown genetic aberrations in the remaining cases of AML will result in distinct gene expression patterns that can be used for classification. Classification by gene expression profiling may reduce the number of cases for which multiple diagnostic procedures (cytomorphology, FISH, RT-PCR, karyotyping) are performed by at least 40%. In order to use gene expression signatures as a new diagnostic tool, prospective studies are needed that determine the feasibility of obtaining sufficient high-quality RNA for successful gene expression profiling in clinical practice. Importantly, gene expression profiles may give more insight into the biology and the pathophysiology of the different subtypes of AML which may then point to new ways to treat these patients more effectively.

Authorship and Disclosures

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